Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone

C. G. Wilkerson *et al.*

Science **344**, 90 (2014);
DOI: 10.1126/science.1250161

Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone

C. G. Wilkerson,[1,2,3]* S. D. Mansfield,[4]* F. Lu,[5,6]* S. Withers,[1,2]† J.-Y. Park,[4] S. D. Karlen,[6] E. Gonzales-Vigil,[4] D. Padmakshan,[6] F. Unda,[4] J. Rencoret,[5,6]‡ J. Ralph[5,6]§

Redesigning lignin, the aromatic polymer fortifying plant cell walls, to be more amenable to chemical depolymerization can lower the energy required for industrial processing. We have engineered poplar trees to introduce ester linkages into the lignin polymer backbone by augmenting the monomer pool with monolignol ferulate conjugates. Herein, we describe the isolation of a transferase gene capable of forming these conjugates and its xylem-specific introduction into poplar. Enzyme kinetics, in planta expression, lignin structural analysis, and improved cell wall digestibility after mild alkaline pretreatment demonstrate that these trees produce the monolignol ferulate conjugates, export them to the wall, and use them during lignification. Tailoring plants to use such conjugates during cell wall biosynthesis is a promising way to produce plants that are designed for deconstruction.

Lignin is a complex phenolic polymer that is essential for plant growth and development but concurrently acts as a major impediment to industrial processing. Research efforts globally have therefore focused on altering the natural lignification processes to produce plants with cell walls that process more readily, for example, to liberate carbohydrates with minimal inputs (*1*–*5*).

The biosynthetic steps to produce the monomers used in the synthesis of lignin have been elucidated (*1*, *4*, *5*), although new genes continue to be discovered (*6*, *7*), and several transcription factors integral to controlling the lignin biosynthetic network have been identified (*8*). It was also empirically discovered that some perturbations led to the synthesis of lignins that incorporated alternative monomers, usually derived from products of incomplete monolignol biosynthesis, spawning the idea that lignins could be designed to encompass substantial structural alterations that would engender unique properties (*4*, *9*, *10*).

Studies of natural plant tissues, along with those from mutants and transgenics with mis-

[1]Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA. [2]Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI 48824, USA. [3]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA. [4]Department of Wood Science, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada. [5]Department of Biochemistry, University of Wisconsin–Madison, Madison, WI 53706, USA. [6]U.S. Department of Energy (DOE) Great Lakes Bioenergy Research Center, the Wisconsin Energy Institute, University of Wisconsin–Madison, Madison, WI 53726, USA.

*These authors contributed equally to this work.
†Present address: Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA.
‡Present address: Instituto de Recursos Naturales y Agrobiología de Sevilla, Consejo Superior de Investigaciones Científicas, Post Office Box 1052, E-41080 Seville, Spain.
§Corresponding author. E-mail: jralph@wisc.edu

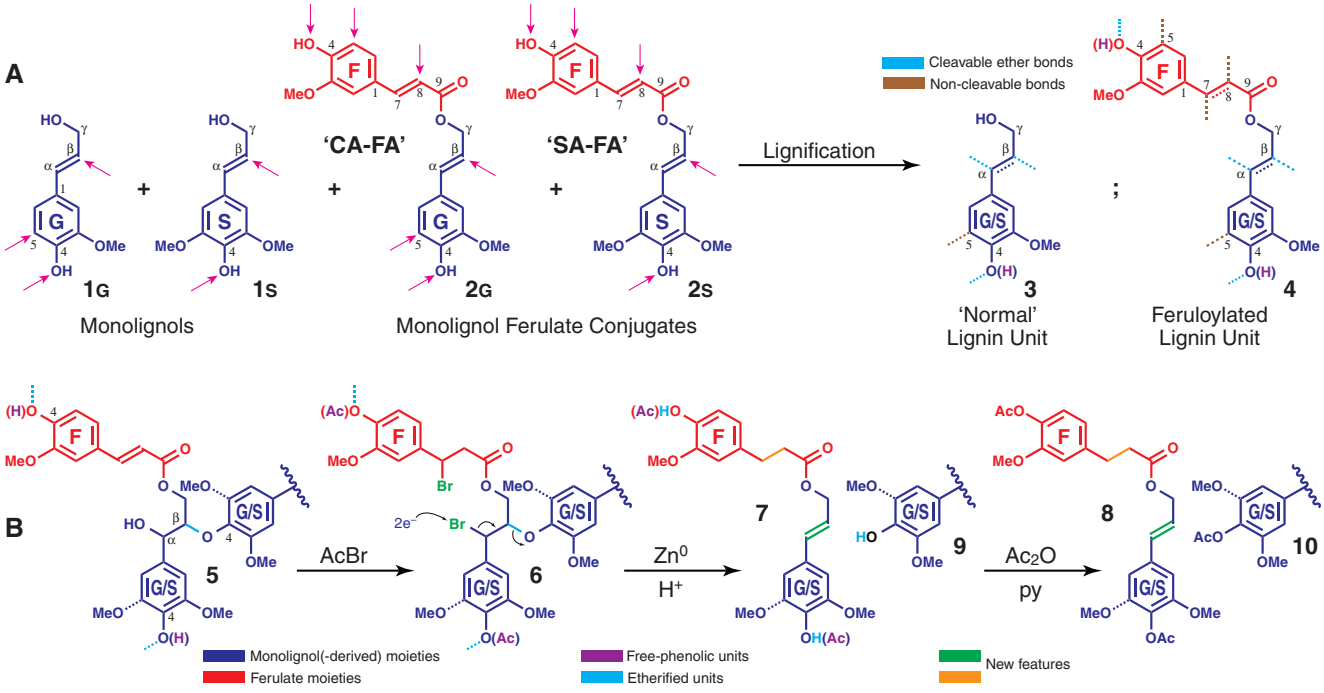

Fig. 1. General schemes for lignification and a method to provide evidence for monolignol ferulate conjugate incorporation into lignins. (**A**) Lignification of monolignols **1** by combinatorial radical cross-coupling reactions produces a lignin in which the units can be represented by generic structure **3**. Analogous lignification of monolignol ferulate conjugates **2** produces structural units **4** in which lignin moieties are γ-acylated by ferulate. Sites of radical coupling for the monolignols **1** and the conjugates **2** are indicated by magenta arrows. In the final lignin units **3** and **4**, cleavable ether bonds are indicated by dotted bonds in cyan; noncleavable bonds, in brown. The complexity with which the conjugates are incorporated is illustrated in fig. S2. Ac, acetyl group; py, pyridine; Me, methyl group; CA-FA, coniferyl ferulate; SA-FA, sinapyl ferulate. (**B**) The DFRC method releases conjugates **8** that diagnostically result from structures **5** within the lignin; the crucial double bond (colored green in **8**) arises only upon cleavage of the signature lignin β-ether bonds, and the ferulate moiety remains attached to its parent unit. Thus, the DFRC method releases an acetylated dihydroferulate analog **8**, of the monolignol ferulate conjugate **2** that was incorporated into the lignin, via reactions that specifically cleave lignin β-ethers but leave the γ-esters intact. Because the initially free-phenolic versus etherified units are acetate-tagged differentially at the stage of intermediate **7**, acetylation with perdeuteroacetic anhydride in the final step can fully reveal the etherification profile of the released units as they were in the cell wall (fig. S8).

regulated monolignol biosynthetic genes, have led to some remarkable discoveries, including plants that produce homopolymers from a range of traditional [e.g., *p*-coumaryl and sinapyl alcohols (*11*, *12*)] as well as nontraditional monomers [e.g., caffeyl and 5-hydroxyconiferyl alcohols and the hydroxycinnamaldehydes (*13*–*16*)]. These observations illustrate the inherent pliability of the lignification process (*4*, *10*, *17*). Therefore, the formal design of an improved polymer using unconventional monomers seems to be a feasible path to tailor plants with superior processing properties for both paper and biofuels production (*9*, *10*).

To that end, the introduction of monolignol ferulate conjugates **2** (Fig. 1 and fig. S1) into the lignin monomer pool appears to be one of the most promising. These exotic conjugates have been shown, but to date only in in vitro model systems, to be capable of introducing readily cleavable ester bonds into the lignin backbone, permitting easier depolymerization (*10*, *18*). Three lines of independent research precipitated the idea that it would be possible to engineer plants to produce such conjugates for lignification. First, although plants have not been shown to use monolignol ferulate conjugates for lignification, all grasses use analogous monolignol *p*-coumarate conjugates (Fig. 1, *p*-coumarate analogs of **2** without the F-ring methoxyl) (*10*, *19*). However, because of their preference for radical transfer over radical coupling (*10*), *p*-coumarates do not polymerize into growing lignin chains and consequently remain almost entirely as free-phenolic pendent entities. Second, unlike the *p*-coumarate moiety, the ferulate, with an additional methoxyl group, is compatible with normal lignification reactions and can integrally incorporate into the polymer (*10*). Polysaccharide-lignin cross-linking results in grasses where the arabinoxylan-bound ferulates (or the diferulates that derive from them by radical dehydrodimerization) radically cross-couple with lignin monomers and higher oligomers during lignification (*10*). The full compatibility of ferulate with lignification leads to complex homo- and cross-coupling (fig. S2) that renders its products difficult to detect in the lignin polymer and makes currently impossible the accurate quantification of the extent of its incorporation (*10*). Because the monolignol ferulate conjugate has two phenolic moieties, it incorporates in a manner that produces ester bonds in the lignin backbone (as schematically shown in Fig. 1 and fig. S1). Third, the substantial research characterizing natural plant lignins, a range of monolignol biosynthetic pathway mutants, and transgenic plants with misexpressed biosynthetic genes revealed that the process of lignification is metabolically plastic; various nonmonolignol phenolic monomers have been shown to actively participate in lignification (*3*–*5*, *9*, *10*, *17*). In fact, because of the combinatorial chemical nature of lignification and the established theory that the polymerization process is not protein- or enzyme-mediated (*17*), a phenolic compound proximal to lignifying tissue may incorporate into lignin subject to its chemical compatibility—its ability to form radicals and to couple and cross-couple with the available phenolics. If a plant could therefore synthesize the monolignol ferulate conjugates **2** with the appropriate temporal and spatial control and had the ability to transport them to the developing wall, the conjugates would ultimately incorporate into the growing lignin polymer (*10*). Recent studies using a corn cell

Table 1. Kinetic data for AsFMT purified from *E. coli*. Michaelis constant (K_m) and specific activity (V_{max}) data are calculated from the mean of at least three replicates ± SE. There was essentially no detectable activity with *p*-coumaroyl-CoA, so data are not given. K_{cat} is the catalysis rate; 1 nkat = 1 nMol product per second.

Varying substrate	Saturating substrate	K_m ± SE (μM)	V_{max} ± SE (nkat mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})
Feruloyl-CoA	Coniferyl alcohol	0.97 ± 0.14	8547 ± 144	426	438.81
Coniferyl alcohol	Feruloyl-CoA	182 ± 20	8060 ± 298	401	2.21
Sinapyl alcohol	Feruloyl-CoA	204 ± 31	2212 ± 118	110	0.54
p-Coumaryl alcohol	Feruloyl-CoA	373 ± 43	14540 ± 721	724	1.94

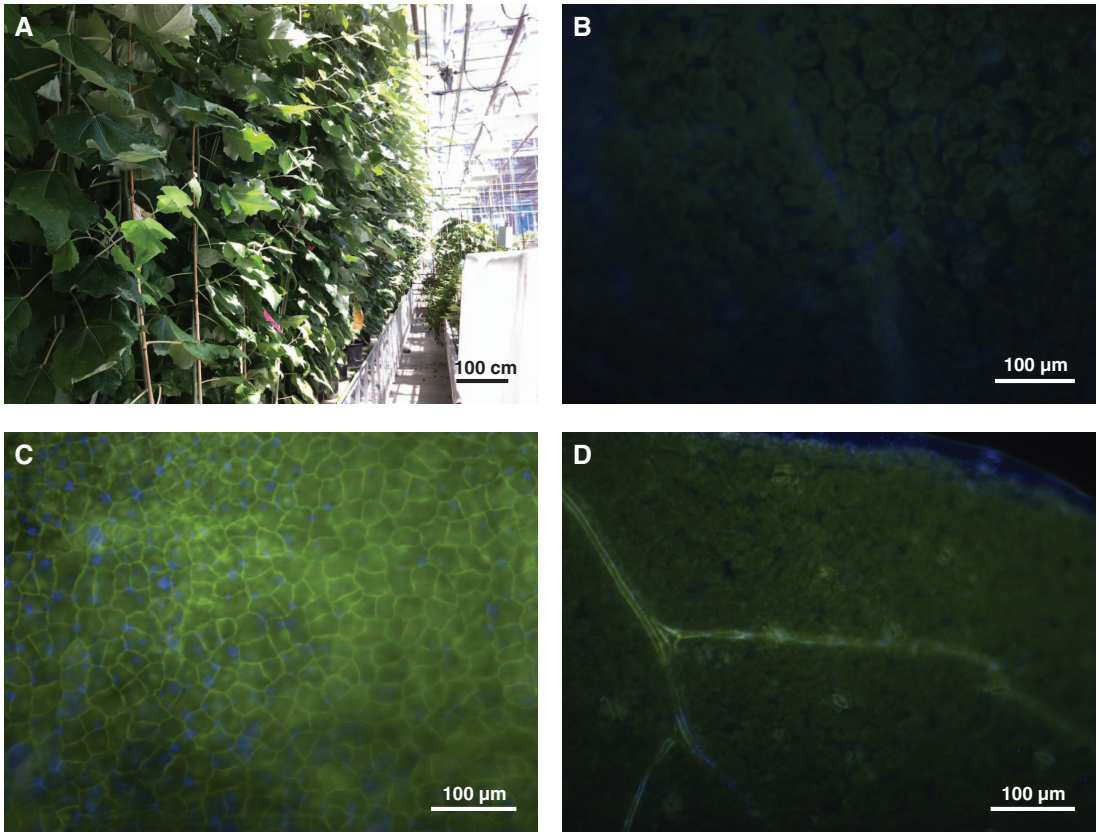

Fig. 2. Images showing YFP-tagged FMT protein in poplar leaves from transgenics in which the *A. sinensis FMT* gene was driven by the universal 35S or the poplar xylem-specific *CesA8* promoter. (**A**) Poplar FMT transgenics growing in the greenhouse do not display any visible phenotypic differences from WT. (**B**) Non-transgenic control line. (**C**) *35S::FMT* showing universal FMT expression. (**D**) *CesA8::FMT* line showing vascular-tissue–specific FMT expression.

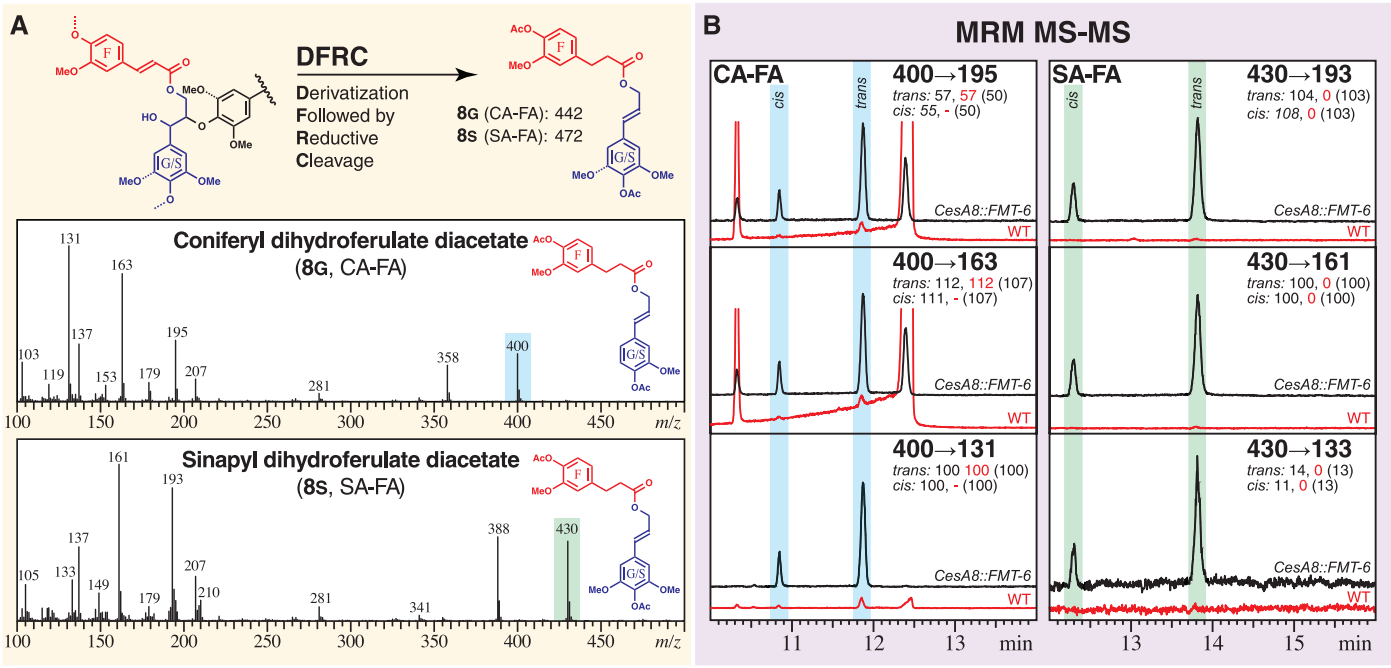

Fig. 3. Mass spectrometric evidence for the incorporation of monolignol ferulate conjugates into transgenic poplar lignins. (**A**) DFRC releasable conjugate **8**; Mass spectra for synthetic compounds **8G** and **8S** are from Q3 scans on a GC-triple-quad MS: molecular ions [mass/charge (*m/z*) 442, 472] lose ketene (*m/z* = 42) to yield the base peak (fig. S8) (*m/z* = 400, 430). (**B**) Triple-quad multiple reaction–monitoring (MRM) chromatograms of the poplar-derived *m/z* = 400 parent ion to the diagnostic 195, 163, and 131 product ions and *m/z* = 430 parent ion to the 193, 161, and 133 product ions; the numbers for the cis and trans isomers are the MRM intensities for transgenic *CesA8::FMT-6* in black and WT in red (with the corresponding measured intensities from the synthetic standards in parentheses) relative to the 400→131 (CA-FA) and 430→161 (SA-FA) peak for each. All of the data for the poplar-released conjugates match (by retention times, mass spectra, collision-induced fragmentation, and MRM relative intensities of daughter ions), providing evidence for the release of the diagnostic conjugates from the lignin in transgenic poplars. The released DFRC conjugate **8G** derives, in part, from units **5** (Fig. 1) in which the phenolic ends of the CA and the FA moiety are further etherified (fig. S8).

wall synthetic model system demonstrated the feasibility of incorporating such conjugates into lignin and supported the need to implement such a strategy in planta (*18*).

The first requirement was to obtain the gene coding for a protein or enzyme capable of catalyzing the formation of the conjugate, a feruloyl–coenzyme A (CoA) monolignol transferase (FMT). Evidence for the existence of such a gene was found in the Chinese medicinal *dong quai* or Chinese angelica (*Angelica sinensis*) in which the root tissue inherently contained ~2 weight % coniferyl ferulate (*20*). Deep expressed sequence tag sequencing led to identification of many BAHD transferases (table S1). The second most abundant transcript (phylogenetic tree in fig. S3) had only moderate similarity to any putative *Arabidopsis* gene (*Arabidopsis* is not known to make such ferulate conjugates) and ultimately produced a recombinant protein in *Escherichia coli* (fig. S4) with the desired transferase activities, namely high activity with feruloyl-CoA and low activity with *p*-coumaroyl–CoA (Table 1). Such selectivity ensures that the enzyme would not use *p*-coumarate to produce monolignol *p*-coumarate conjugates that, as we know from grasses, do not introduce ester bonds into the lignin backbone. Additionally, the enzyme could use any of the three monolignols as a target substrate (Table 1).

A key additional step consisted of expressing the gene spatially and temporally in the tissues that form lignin. This was successfully achieved in hybrid poplar (*Populus alba* × *grandidentata*) by using both a universal 35*S* promoter and the poplar *CesA8* xylem-specific promoter involved in secondary cell wall cellulose biosynthesis. Fusion with a yellow fluorescent protein (YFP) reporter gene demonstrated that the protein was indeed being produced and facilitated localization for each promoter used. Examination of leaf extracts showed that, unlike in the control plants, FMT protein was being produced in planta in many tissues in the case of the ubiquitous promoter and only in the leaf vasculature (xylem) when the *CesA8* promoter was used (Fig. 2). We subsequently isolated the FMT protein from these plants and demonstrated in vitro that it was able to produce the target conjugates from its substrates, feruloyl-CoA and a monolignol (figs. S5 and S6). In all cases, the plants showed no phenotypic growth abnormalities in the greenhouse (Fig. 2). The total lignin levels generally remained the same as in wild-type (WT) controls and displayed only a very slight consistent increase in the syringyl-to-guaiacyl (S/G) ratio (table S2).

Unequivocally determining that these plants are capable of incorporating monolignol ferulates into the lignin polymer is particularly challenging because ferulate is naturally integrally incorporated into lignins by combinatorial radical coupling, the complexity of which is illustrated in fig. S2. However, derivatization followed by reductive cleavage (DFRC) is capable of cleaving the lignin-signature β-ether bonds while leaving γ-esters intact and has previously been used to analyze *p*-coumaroylated lignins (*21*). With modifications to this method, we were able to show that the novel monolignol ferulates, coniferyl ferulate **2G** and sinapyl ferulate **2S** (Fig. 1), are indeed incorporated into the lignin of the transgenic trees. Thus, a small fraction of the expected structures **5** in the polymer that would result from incorporating monolignol ferulate conjugates into lignins (Fig. 1 and fig. S2) can be cleaved, releasing monolignol (dihydro)ferulate conjugates **8** that are quantifiable by gas chromatography–mass spectrometry (GC-MS) (Figs. 1B and 3). The double bond in the monolignol moiety of **8**, as in the normal DFRC monomers, arises only from reductively cleaving the β-ether bond. Compounds **8** are therefore diagnostic markers indicating that the monolignol ferulate conjugate **2** has incorporated into the polymer by radical coupling. Structures **5** (Fig. 1B) are only a small fraction of the many structures (represented by the generic structure **4**) that can result when the combinatorial radical coupling reactions incorporate monolignol ferulates into lignin (fig. S2), but they are the only com-

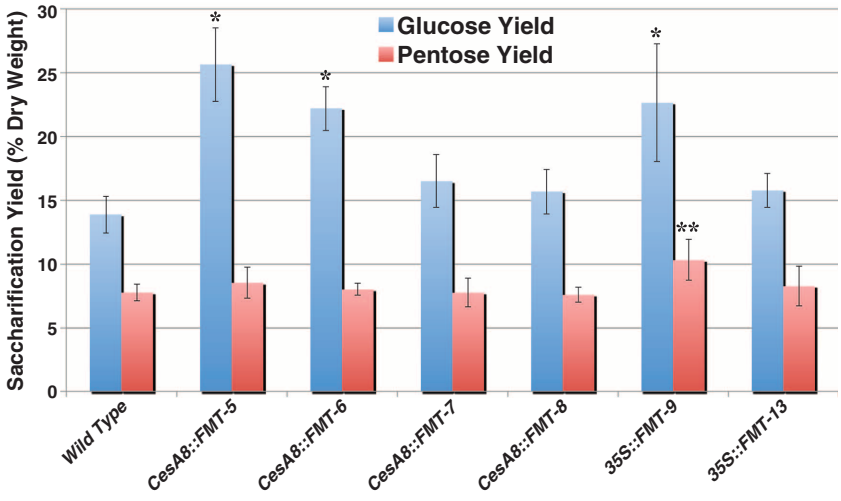

Fig. 4. Digestibility data on various mild alkaline–pretreated transgenic poplar lines compared to WT. Error bars indicate SD from the mean of triplicate determinations; $*P < 0.01$; $**P < 0.005$.

ponents that can generate the diagnostic product **8** (Fig. 3). The methodology does, however, permit confirmation that the trees have incorporated monolignol ferulates, at levels markedly above those of WT, into the lignin polymer by the radical coupling reactions that typify lignification (Fig. 1A). It also indicates that, like the natural monolignol *p*-coumarate analogs in grasses, these nonnative monolignol ferulate conjugates are transported intact to the lignifying zone. The GC-MS evidence (Fig. 3) that our transgenic poplars, particularly lines with the *FMT* gene driven by the xylem-specific *CesA8* promoter, are performing all of the biochemistry and chemistry desired is therefore compelling.

A key feature that allows the ensuing modified lignin to be depolymerized under mild conditions is the incorporation of the ferulate unit into the lignin backbone rather than simply existing as a pendant unit. Further insight into how well the conjugates are incorporated was revealed following an isotopically-labeled-reagent modification to the DFRC method (*22*). As delineated in fig. S8, 53% of the ferulate and 53% of the coniferyl alcohol moieties in the releasable conjugates were from phenol-etherified units, and 36% resulted from units that have both ends of the conjugate etherified. Because only a small fraction of units can release the conjugate and because the double bond in the coniferyl alcohol moiety implies that it is derived from a β-etherified unit, an etherified phenolic level comparable to that of guaiacyl monomers released by DFRC, determined in the same runs here at ~50%, confirms that the conjugate is incorporated into the lignin polymer about as well as a conventional monolignol.

Quantifying the level of conjugate incorporation is also a challenge. Currently, no method exists for the analogous quantification of the arabinoxylan-bound ferulates that are incorporated into grass lignins, and it has remained extremely difficult to even detect them, for example, by nuclear magnetic resonance (NMR), decades after their discovery in part because of the huge range of combinatorial products (illustrated in fig. S2). An estimate (fig. S9 and table S4) can be made by using a model system in which isolated cell walls were ectopically lignified with coniferyl alcohol and 0, 20, 40, and 60% coniferyl ferulate (*18*). By plotting the release of the DFRC conjugate in the model system and those released from the current transgenic lines, we estimate that the *CesA8::FMT* poplar trees are incorporating ~7 to 23% of the ferulate conjugates **2** into their lignins.

The utility of lignins altered in such a fashion is ultimately determined by how well the ensuing cell walls containing these specialized conjugates can be processed industrially. Substantial improvements in saccharification after mild alkaline (6.25 mM NaOH, 90°C, 3 hours) pretreatment (*23*) are evident in the lines determined to contain high levels of transcripts, proteins, and lignified ferulate conjugates (Fig. 4).

The initial concept of inducing plants to use monolignol ferulate conjugates during lignification is realized here; our approach appears to be capable of producing plant materials that are designed for deconstruction. In a fundamental sense, it supports the idea that we can exploit the inherent metabolic plasticity of lignification (*4*) to alter lignins for societal benefit by inducing plants to synthesize novel phenolic monomers that are compatible with inherent plant biochemistry and development. The strategic introduction of ester bonds into the backbone of the lignin polymer is an advance that portends the production of crop plants in which cell wall deconstruction can be realized with reduced energy and/or chemical inputs.

References and Notes

1. X. Li, J. K. Weng, C. Chapple, *Plant J.* **54**, 569–581 (2008).
2. F. Chen, R. A. Dixon, *Nat. Biotechnol.* **25**, 759–761 (2007).
3. R. Vanholme, K. Morreel, J. Ralph, W. Boerjan, *Curr. Opin. Plant Biol.* **11**, 278–285 (2008).
4. J. Ralph *et al.*, *Phytochem. Rev.* **3**, 29 (2004).
5. W. Boerjan, J. Ralph, M. Baucher, *Annu. Rev. Plant Biol.* **54**, 519–546 (2003).
6. R. Vanholme *et al.*, *Science* **341**, 1103 (2013).
7. S. Withers *et al.*, *J. Biol. Chem.* **287**, 8347–8355 (2012).
8. E. Li *et al.*, *New Phytol.* **194**, 102–115 (2012).
9. R. Vanholme *et al.*, *New Phytol.* **196**, 978–1000 (2012).
10. J. Ralph, *Phytochem. Rev.* **9**, 65–83 (2010).
11. J. J. Stewart, T. Akiyama, C. Chapple, J. Ralph, S. D. Mansfield, *Plant Physiol.* **150**, 621–635 (2009).
12. N. D. Bonawitz *et al.*, *Nature*, 10.1038/nature13084 (2014).
13. F. Chen, Y. Tobimatsu, D. Havkin-Frenkel, R. A. Dixon, J. Ralph, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1772–1777 (2012).
14. R. Vanholme *et al.*, *Plant J.* **64**, 885–897 (2010).
15. J.-K. Weng, H. Mo, C. Chapple, *Plant J.* **64**, 898–911 (2010).
16. Q. Zhao *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 13660 (2013).
17. J. Ralph *et al.*, in *Recent Advances in Polyphenol Research*, F. Daayf, A. El Hadrami, L. Adam, G. M. Ballance, Eds. (Wiley-Blackwell, Oxford, 2008), vol. 1, pp. 36–66.
18. J. H. Grabber, R. D. Hatfield, F. Lu, J. Ralph, *Biomacromolecules* **9**, 2510–2516 (2008).
19. D. L. Petrik *et al.*, *Plant J.* **77**, 713–726 (2014).
20. J. J. Xie *et al.*, *Molecules* **14**, 555–565 (2009).
21. F. Lu, J. Ralph, *J. Agric. Food Chem.* **47**, 1988–1992 (1999).
22. P. T. Martone *et al.*, *Curr. Biol.* **19**, 169–175 (2009).
23. N. Santoro *et al.*, *Bioenergy Res.* **3**, 93–102 (2010).
24. Y. Zhu *et al.*, *RSC Adv.* **3**, 21964 (2013).
25. J. Ralph, J. H. Grabber, R. D. Hatfield, F. Lu, U. S. Patent 8,569,465 B2 (2013).
26. C. G. Wilkerson, J. Ralph, S. Withers, U.S. Patent 2013/0219547 (2013).
27. C. G. Wilkerson, J. Ralph, S. Withers, S. D. Mansfield, U.S. Patent 2013/0203973 (2013).

Acknowledgments: This work was funded by the DOE Great Lakes Bioenergy Research Center (DOE Basic Energy Research Office of Science DE-FC02-07ER64494). We also thank Y. Zhu, M. Regner, and H. Kim for making some of the required conjugates as described (*24*); S. Liu for sample preparation; N. Santoro for the digestibility data determinations; and the original team, including J. Grabber and R. Hatfield, who embarked with us on the idea and demonstrated its potential in models systems as described (*18*). The redesigned polymer presented herein has become known as Zip-lignin; the method for plant modification and the use of the gene for altering plants have been patented (*25–27*). *Angelica sinensis* plants were obtained with the help of Joe Hollis at Mountain Garden Herbs in Burnsville, North Carolina. The DNA sequence for the *AsFMT* gene (see supplementary materials) has been deposited in GenBank under the accession number JA758320.1 (www.ncbi.nlm.nih.gov/nuccore/JA758320.1). Materials and methods, other figures, and further data are reported in the supplementary materials. Future use of materials involved with these projects is governed by material transfer agreements, available from Lauren Meyers, (Wisconsin Energy Institute, lmeyers@energy.wisc.edu) or from the University of Wisconsin's Office of Industrial Partnerships (oip@gran.wisc.edu).

Supplementary Materials

www.sciencemag.org/content/344/6179/90/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S9
Tables S1 to S4
References (*28–39*)

24 December 2013; accepted 12 March 2014
10.1126/science.1250161